Immunity, Vol. 7, 461–472, October, 1997, Copyright ©1997 by Cell Press

Minors Held by Majors: The *H13* Minor Histocompatibility Locus Defined as a Peptide/MHC Class I Complex

Lisa M. Mendoza,[1] Pedro Paz,[1] Aamir Zuberi,[2] Greg Christianson,[2] Derry Roopenian,[2] and Nilabh Shastri[1]*

[1]Division of Immunology, LSA 421
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720

[2]The Jackson Laboratory
Bar Harbor, Maine 04609

Summary

The products of minor histocompatibility (*H*) loci are serious barriers to tissue transplantation even among major histocompatibility complex (MHC) identical individuals, frequently causing chronic graft rejection and graft versus host disease. Over 50 minor *H* loci map to mouse autosomal chromosomes but none are known at the molecular level. By expression cloning, we identified the *H13* locus, a classical minor *H* locus first detected 30 years ago by the trait of graft rejection. The $H13^a$ allele is located on chromosome 2 and encodes a novel protein that yields the rare naturally processed nonapeptide SSVVGVWYL (SVL9) for presentation by the D^b MHC class I molecule. The SVL9 peptide binds D^b MHC despite the absence of the consensus binding motif, and a conservative methyl group substitution (Valine 4 ↔ Isoleucine) explains why reciprocal T cell responses are elicited in $H13^a$ and $H13^b$ congenic strains.

Introduction

Minor histocompatibility (H) antigens cause graft versus host disease (GVHD) and chronic graft rejection in a large percentage of transplantation cases, even when the donor and recipient tissues share the same major histocompatibility complex (MHC) antigens (Goulmy et al., 1996). The genetic polymorphisms responsible for minor H antigens have been studied for over 80 years (Little, 1914) and have resulted in the delineation of over 50 autosomal and sex-linked loci based upon their inheritance and segregation patterns in recombinant and congenic mice (Little, 1941; Snell, 1948; Doolittle et al., 1996). It is very likely that the significant clinical problems associated with tissue graft rejection result from the existence of a similar number of *H* loci in humans. The minor H antigens do not normally induce antibody responses but rather serve as the source of processed peptides presented by MHC molecules to T cells (Wallny and Rammensee, 1990). The rejection phenotype conferred by minor *H* loci is thus the result of a T cell response elicited by qualitative or quantitative differences in the antigenic peptide display of the MHC molecules on the donor and/or host cell surface.

*To whom correspondence should be addressed.

Interest in minor *H* loci has endured not only because they are relevant to clinical transplantation but also because their antigens have served as important models to understand the role of self and foreign peptides on the thymic and peripheral T cell repertoire (Bevan, 1975; von Boehmer and Kisielow, 1990; Auchincloss and Sultan, 1996; Goulmy, 1996). Why minor *H* loci serve as transplantation barriers is not well understood. Possible explanations include their influence on antigen processing and presentation, on the development of the T cell repertoire, or on peripheral immune responsiveness (Loveland and Lindahl, 1991; Roopenian, 1992). For example, the minor *H* alleles could differ in their transcriptional regulation, leading to the presence or absence of the antigen precursor protein and as a result the processed H peptide/MHC complex itself. Differences in peptide/MHC display, despite equivalent expression, could also result from variations in the suitability of the polymorphic donor proteins for the antigen processing pathway (York and Rock, 1996). Alternatively, structurally related allelic H peptides could act as altered agonist/antagonists and influence the peripheral T cell responsiveness or T cell development in the thymus (Allen, 1994; Jameson and Bevan, 1995; Jameson et al., 1995). Determining which of these or even more complex hypotheses account for the immunobiology of minor H antigens requires the identification of their genes and processed peptide products.

Minor *H* antigens can in principle be identified by positional cloning techniques wherein detailed genetic information of the defined chromosomal location leads to their discovery. Indeed knowledge of the mitochondrial origin of maternally transmitted H antigens did lead to the discovery of the antigenic precursors as the ND1, COI, and ATPase6 proteins from among the 13 encoded by the mitochondrial genome (Loveland et al., 1990; Morse et al., 1996; Bhuyan et al., 1997). The N-formylated peptides from these proteins were presented by the nonpolymorphic H2-M3 MHC class 1b molecule and other peptides by the conventional MHC class I molecules (Dabhi and Lindahl, 1996; Bhuyan et al., 1997). Likewise, prior knowledge that the *HY* loci mapped to the Y chromosome facilitated the identification of the *Smcy* and *Uty* genes as the source of the mouse male-specific *H* antigens (King et al., 1994; Scott et al., 1995; Greenfield et al., 1996). Positional cloning approaches are, however, orders of magnitude more difficult for the gene-rich autosomal chromosomes that harbor the great majority of minor *H* loci.

Biochemical purification of the antigenic peptide provides an alternative approach to identifying T cell–stimulating peptides (Falk et al., 1991b; Jardetzky et al., 1991; Hunt et al., 1992). This peptide purification/mass spectrometry approach, in combination with prior knowledge of DNA sequence, was successful in identifying the HY peptides from the human homolog of the *Smcy* gene (Wang et al., 1995b; Meadows et al., 1997). Similar methods were also successful in identifying a peptide recognized by a T cell obtained from a GVHD patient (den Haan et al., 1995) and another peptide that could induce GVHD in mice (Perreault et al., 1996). However,

the donor proteins for either of these two antigenic peptides are unknown. Whether these antigens represent products of polymorphic *H* loci therefore remains obscure, as does the molecular mechanism of their antigenicity.

Here we apply an expression cloning strategy to define the classical autosomal mouse minor histocompatibility locus, *H13*, at the molecular level. This locus was originally detected by Snell three decades ago as causing tissue incompatibility between inbred mouse strains (Snell et al., 1967). Our strategy allows screening of a cDNA library in recipient cells with lacZ-inducible, antigen-specific T cells as a specific probe for expression of the appropriate peptide/MHC complex (Karttunen et al., 1992; Malarkannan et al., 1995a; Shastri, 1996). The *H13*^{a} cDNA encodes a previously unknown protein that yields the naturally processed nonapeptide SSVVGVWYL (SVL9) as the minor histocompatibility antigen bound to the D^b MHC molecule. Intriguingly, the *H13*^{a} SVL9 peptide does not conform to the D^b MHC consensus binding motif and differs from its *H13*^{b} homolog SSVIGVWYL (SIL9) by a single conservative methyl group substitution. Nevertheless, both SVL9 and SIL9 peptides bind D^b MHC and are detected as dominant antigens in reciprocal anti-H13^a and anti-H13^b immunizations. These findings provide new insights into the nature and immunological role of naturally processed minor H peptides.

Results

Derivation of the H13^a-Specific lacZ Inducible T Cell Hybrid

The CTL line 30NX/B10-1 was obtained from B10.CE (30NX)-*H13*^{b} mice immunized with spleen cells from the congenic C57BL/10 (B10, *H13*^{a}) mice (Cariappa et al., 1996). These two strains differ in a segment of chromosome 2 that includes the *H13* locus (Graff et al., 1977). This cytotoxic T lymphocyte (CTL) line recognizes the H13^a antigen in association with the D^b MHC molecule (data not shown). As a first step toward identification of the antigen, the CTLs were fused with the BWZ.36/CD8 fusion partner to obtain the β galactosidase (lacZ)–inducible hybrid designated 30NX/B10Z (Sanderson and Shastri, 1994). The lacZ response in the hybridoma was induced by splenic blasts of C57BL/6 (B6) mice, or by EL4 cells, a thymoma cell line of B6 origin. The response was inhibited by anti-D^b but not anti-K^b monoclonal antibody (MAb; Figures 1A and 1B, left panels). As a reciprocal control, the anti-K^b, but not anti-D^b, MAb inhibited the response of F1/5R-5Z T cells that recognize a K^b-bound peptide (Figure 1B, right panel). The 30NX/B10Z cells did not respond to blasts from the *H13*^{b} (BALB/c × BALB. B)F1 (H-2^{dxb}) mice, even though these blasts were fully capable of stimulating another T cell hybrid, 18.5Z (Figure 1A). The 30NX/B10Z T cell hybrid therefore displayed the same *H13*^{a}-specific, D^b-restricted reactivity as did the parent CTL line.

Expression Cloning the H13 cDNA

The cDNA clone 47c1, encoding the D^b-restricted antigenic activity, was isolated by expression cloning. The

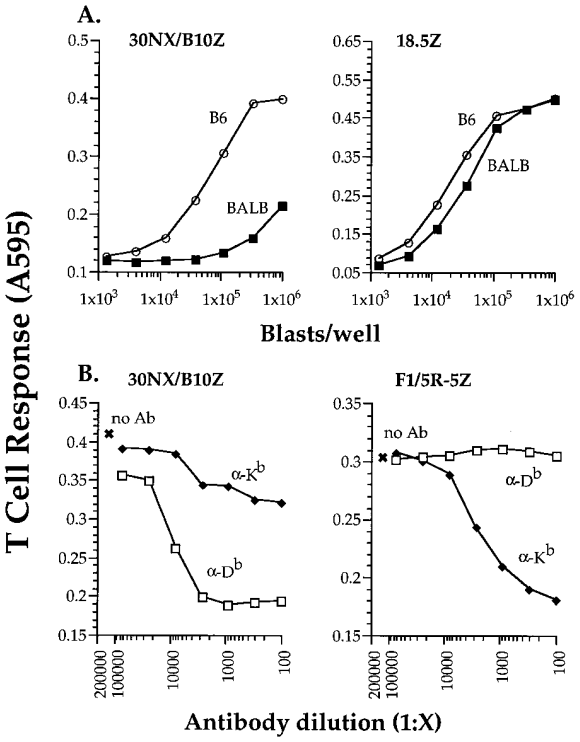

Figure 1. The 30NX/B10Z T-Cell Hybrid Recognizes a D^b MHC-Associated Polymorphic Antigen Expressed in the B6, but Lacking in the BALB Background

(A) B6 or (BALB/c × BALB.b)F1 spleen cells were cultured with ConA and LPS for 48 hr. After three washes, the indicated number of blast cells were cocultured overnight with 1 × 10^5 30NX/B10Z or the control K^b-restricted 18.5Z T cell hybrids. The T cell response was measured as the lacZ activity by conversion of the substrate chlorophenol red β-pyranoside (CPRG) at 595 nm and 655 nm as reference.

(B) The lacZ response of 30NX/B10Z T cells to the B6-derived EL-4 cell line was tested in the presence of indicated dilutions of anti-D^b MAb, B22.249, or anti-K^b MAb, Y3. As a control, the same two antibodies were included in cultures of the K^b-restricted T cell hybrid F1/5R-5Z and B6 blasts.

lacZ inducible 30NX/B10Z T cell hybrid was used as a probe for expression of the cognate ligand in D^b-expressing recipient cells that were transiently transfected with pools of a plasmid cDNA expression library prepared from the EL-4 cell line (Karttunen et al., 1992; Malarkannan et al., 1995a). Representative data from one of twelve 96-well plates show a cDNA pool with above background T cell–stimulating activity (Figure 2A). Indeed, several active plasmid clones from this pool were obtained after fractionating the mixture into individual colonies (Figure 2B). One of these plasmids, designated 47c1, was further tested for its ability to stimulate the 30NX/B10Z T cells in an antigen-specific and MHC-restricted manner. The recipient cells generated the T cell–stimulating ligand, in a dose-dependent manner, only with the 47c1 plasmid DNA when it was cotransfected with the cDNA encoding the D^b, but not the K^b, MHC molecule (Figure 2C). We conclude that the 47c1 cDNA clone allowed expression of an antigen recognized by the 30NX/B10Z T cell.

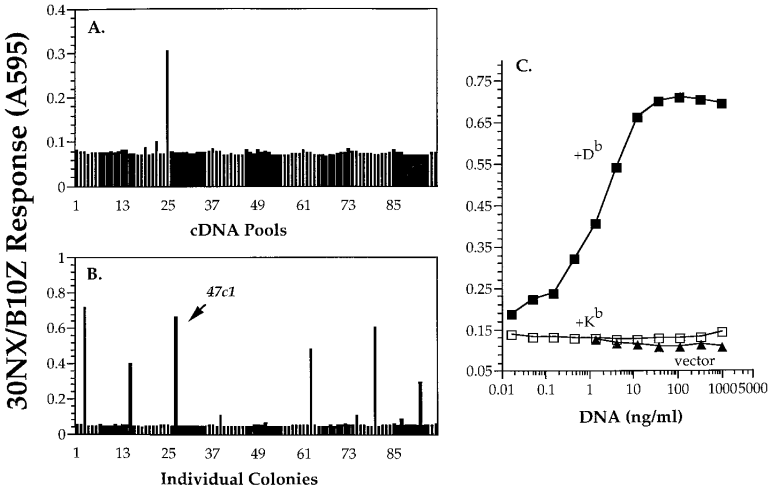

Figure 2. Isolation of the cDNA Clone Encoding the Antigen Presented by the D^b MHC to 30NX/B10Z T Cells

(A) Pools of plasmid DNAs from an EL-4 cDNA library were transiently transfected into LMtk- fibroblasts together with cDNA clones encoding D^b MHC and B7.2 in 96-well plates. Two days later, the 30NX/B10Z T cells were added, and their lacZ response was measured as in the legend to Figure 1. Absorbance data are shown for one of twelve 96-well plates screened.

(B) Response of T cells to the recipient cells transfected as above but with plasmid DNA from individual colonies isolated from the positive pool. Clone 47c1 was selected for further study.

(C) Recipient LMtk- cells were transfected with varying concentrations of the 47c1 plasmid or the vector DNA together with cDNA encoding either the D^b or K^b MHC molecules.

The 47c1 cDNA Clone Maps to the *H13* Locus on Chromosome 2

Genetic studies of allograft rejection and the analysis of CTL activity in vitro have mapped the *H13* locus to mouse chromosome 2 between a proximal H locus, $H3^b$, and agouti (*a*; Snell et al., 1967; Roopenian et al., 1993; D. R., unpublished data). To distinguish the possibilities that the 47c1 cDNA clone was derived from the H13 antigen gene itself, a gene that regulates H13 antigen expression, or an unrelated gene that encodes a cross-reactive antigen, we performed genetic mapping studies. Southern blot restriction fragment length polymorphism (RFLP) analysis was carried out using mouse strains previously typed for *H13* polymorphisms as well as a panel of chromosome 2 congenic strains. With XbaI-digested DNA, unique RFLP bands hybridizing to the 47c1 probe were detected in the $H13^a$ strain C57BL/10 (B10), ~9 kb, versus the ~14 kb fragment in the $H13^b$ strains (CE/J or LP/J; Figure 3A). The prototypic $H13^b$ congenic strain, B10. CE(30NX)-$H13^b$, which was used to generate the anti-$H13^a$ T cell response, clearly contained the 14 kb RFLP band that was identical in size to the $H13^b$ strains, CE/J and LP/J. Furthermore, B10.LP-congenic strains previously used to segregate the $H3^a$, $H3^b$, and *H13* loci showed an RFLP pattern that was remarkably consistent with the known distribution of *H13* alleles (Graff et al., 1977; Figure 3B).

The 47c1 cDNA was mapped at a higher resolution using DNA samples from an interspecific mouse backcross. Polymerase chain reaction (PCR) primers that amplified the 3′ end of 47c1 revealed a sequence polymorphism (SSLP) that was used to type DNAs from The Jackson Laboratory Backcross Panel (http://www.jax.org/resources/documents/cmdata/). The panel consists of 94 (B6 × SPRET/Ei) × B6 (BSB) and 94 (B6 × SPRET/Ei) × SPRET/Ei (BSS) backcross DNAs previously typed for a large number of genetic markers. As summarized in Figure 3C, the 47c1 cDNA cosegregated with *D2Mit22* in 192 informative backcross mice, hence mapping 47c1 to the interval expected for the *H13* locus defined by the graft rejection trait. Taken together, these results prove that the *H13* locus, represented by the 47c1 cDNA clone, is a mouse chromosome 2 gene that is located within 1.06 ± 1.06 cM of *a* and 0.53 ± 0.53 cM of *D2Mit22.*

Location of the Antigenic Activity within the *H13* cDNA Clone

The minimal sequence required to confer antigenic activity within the 47c1 cDNA was defined by testing the functional activity of deletion constructs. Initially, 3′ deletions were generated in the 47c1 cDNA by PCR. A series of reverse PCR primers, together with the vector-encoded forward primer, were used to amplify DNA fragments lacking the C-terminal codons of the H13 gene. The DNA fragments were recloned into the pcDNAI expression vector, and the plasmid DNAs were tested for their ability to generate the peptide/D^b ligand for the 30NX/B10Z T cells (Figure 4A). As judged by the ability of the DNA constructs to generate the 30NX/B10Z T cell response, the antigenic activity was encoded within or overlapped the 108 nucleotides represented by the ΔR4 and the ΔR8 constructs (Figure 4B). Because the antigenic activity was presented by the D^b MHC molecule that binds peptides with the consensus motif XXXXNXX[I,L,M] (Falk et al., 1991b) and peptides encoded within different translational reading frames can be presented by MHC molecules (Shastri and Gonzalez, 1993; Malarkannan et al., 1995a; Wang et al., 1996), we tested minigene constructs representing the only two 9mer sequences containing either a complete (D^b1) or a partial D^b motif (D^b2; Figure 4A). The D^b1 (nucleotides 65–91) and D^b2 (nucleotides 97–123) motifs are encoded within the second and the first translational reading frames (Figure 5A). However, the activity of both minigene constructs was indistinguishable from the vector alone (Figure 4B), strongly suggesting that the H13 antigenic activity was not represented by a motif-bearing peptide.

We then generated another set of PCR deletions at the 3′ end of the 47c1 clone (Figure 5A). The presence of the antigenic activity in the ΔR7, but its loss in the ΔR11 construct, indicated that the antigenic peptide was either contained entirely within or overlapped the C-terminus of the ΔR7 polypeptide (Figure 5B). Indeed the minigene construct MSVL10, encoding only the 10

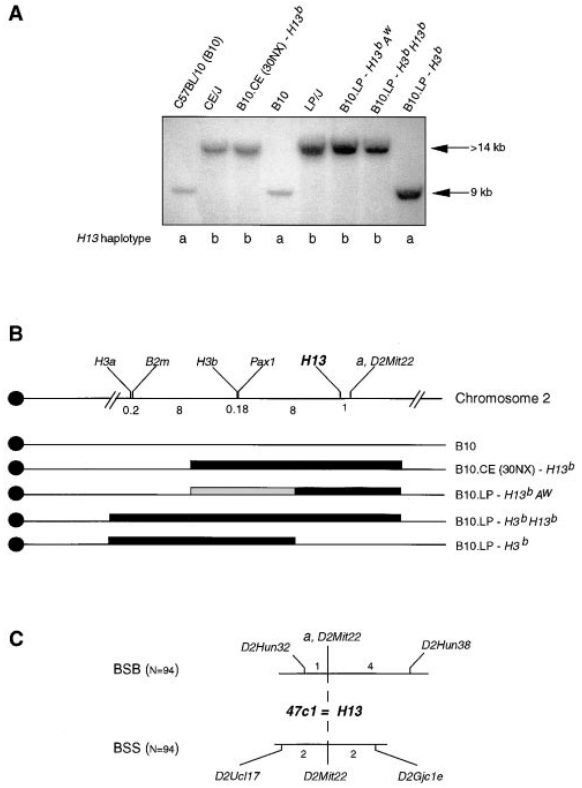

Figure 3. Chromosomal Localization of the 47c1 cDNA

(A) Southern blot RFLP analysis of *H13*^{a} and *H13*^{b} mouse strains. High molecular weight DNA from the mouse strains shown above each lane was digested with XbaI, fractionated, transferred to nitrocellulose filters, and probed with radiolabeled fragment of 47c1 DNA. The 9 kb and the ~14 kb bands segregate with the *a* and *b* alleles of the *H13* locus represented by the mouse strains shown.

(B) Schematic representation of the chromosome 2 region of *H3* and *H13* congenic mice analyzed by RFLP in (A). Black boxes represent congenic donor segments, and open box represents a segment of unknown heritage. Relative locus positions and distances (in cM) are based on earlier mapping studies (Graff et al., 1977; Roopenian et al., 1993) and the current mouse genome database (website http://www.informatics.jax.org/encyclo.html).

(C) Positioning of the 47c1 cDNA relative to other loci on mouse chromosome 2 based on SSLP analysis of The Jackson Laboratory interspecific BSB and BSS backcross panels. N, number of meioses analyzed.

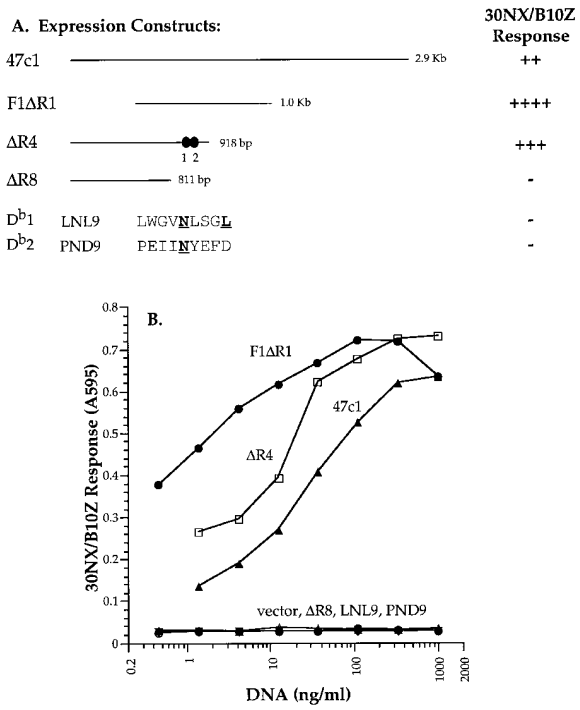

Figure 4. Location of the H13 Antigenic Activity within the 47c1 cDNA Clone Maps to 108 Nucleotides but Not to the Peptides Matching the D^b Consensus Motif.

(A) Schematic of the expression constructs used to locate the 30NX/B10Z T cells stimulating activity within the 47c1 cDNA clone. The lines indicate the location of the deletion fragments relative to the original 47c1 cDNA. The residues shown as D^{b}1 and D^{b}2 are the only two sequences, encoded within the translational reading frames of the antigenically active fragment that resembles the D^b consensus motif XXXX[N]XXX[I,L,M].

(B) T cell responses induced by Db+ recipient cells that were transiently transfected with varying amounts of plasmid DNA of the indicated expression constructs shown in (A). The transfection conditions and measurement of the T cell response were the same as in the legends to Figures 1 and 2.

SSVVGVWYLL residues and an additional methionine codon for translational initiation, was as active as the 47c1 cDNA. However, another MSVL9 (Met-SSVVGVWYL) construct lacking the second C-terminal leucine residue was 100- to 1000-fold more active than either the MSVL10 or the 47c1 cDNA constructs (Figure 5B). Thus, SVL9 rather than SVL10 was likely to represent the naturally processed H13 peptide.

Further direct evidence that the H13 antigenic activity was defined by the SVL9 sequence was obtained with synthetic peptides. Both synthetic SVL9 and SVL10 peptides, representing only the SVVGVWYL and SVVGVWYLL residues respectively, were potent stimulators of the 30NX/B10Z T hybrid in the presence of D^b APC. However, as observed for endogenously generated ligand (Figure 5B), the SVL9 peptide was about 1000-fold more active than the SVL10 peptide in stimulating the 30NX/B10Z T cells (Figure 5C). The ability of SVL9 to stimulate T cells at pico- to femtomolar concentrations is a defining characteristic of naturally processed peptides presented by MHC class I molecules (Rotzschke et al., 1990a; Van Bleek and Nathenson, 1990). In contrast, the same SVL9 peptide failed to stimulate Lpa/2RZ, another *H3a*^{a}-specific, D^b-restricted T cell hybrid at even 100,000-fold higher concentration. Note that, except for the C-terminal leucine residue, the SVL9 peptide bears no resemblance to the consensus motif, XXXX[N]XXX[I,L,M], defined for the D^b MHC molecule (Falk et al., 1991b). We conclude that despite this apparent handicap, the SVL9 peptide simulates the antigenic H13^a/D^b complex recognized by the 30NX/B10Z T cells.

To define the donor protein for the H13 antigenic peptide, ~1.5 kb of available sequence of the 47c1 cDNA clone was compared with the nonredundant and expressed sequence tag (EST) data bases at the NCBI GenBank DNA repository. No significant similarities were found for either the nucleotide or the predicted amino acid sequence of 47c1 cDNA in the nonredundant

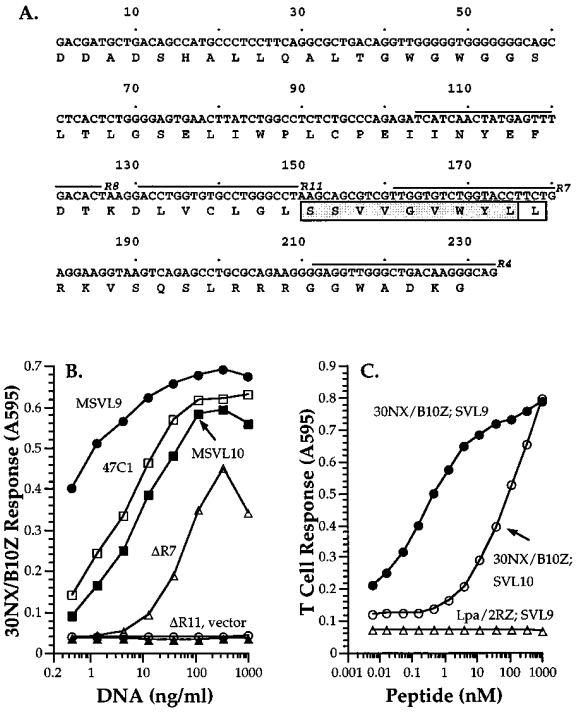

Figure 5. Identification of the Anti-H13^a T Cell–Stimulating Activity as the SSVVGVWYL Nonapeptide

(A) Nucleotide sequence of the antigenic 47c1 fragment and its predicted translation (accession number AF017785). The lines above the sequence show the location of the reverse PCR primers (R4, R7, R8, and R11) used to generate deletions within this region. The residues of the SVL10 and SVL9 peptides are enclosed within boxes and shaded.

(B) The T cell response to APC transfected with the indicated expression constructs. The MSVL10 and MSVL9 constructs encode the SVL10 and SVL9 residues with a methionine codon for translational initiation.

(C) The response of H13^a-specific 30NX/B10Z and H3a^a-specific Lpa/2RZ T cells to the indicated concentration of SVL9 and SVL10 synthetic peptides added to D^b-L cells. The conditions for transfection and measurement of T cell responses were the same as in the legends to Figures 1 and 2.

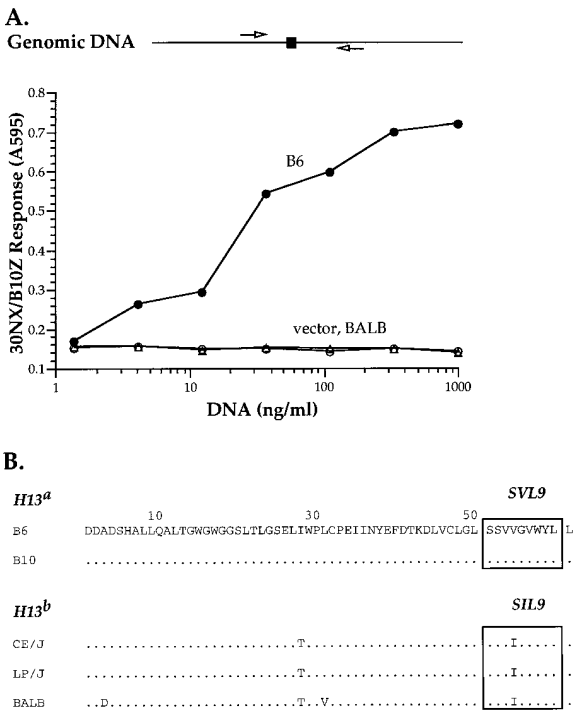

Figure 6. Analysis of the Allelic Polymorphism at the *H13* Locus

(A) The PCR expression constructs were generated by amplifying genomic DNA between the indicated forward and reverse primers. Constructs from the B6-*H13*^{a} and BALB *H13*^{b} strains were transfected into D^{b+} recipient cells, which were then tested as APC for stimulating the 30NX/B10Z T cell response. Data shown are representative of one of 18 B6 and 5 BALB PCR constructs tested.

(B) The predicted amino acid sequences of the antigenic regions of the B6, B10 (H13^a) versus the CE/J, LP/J and BALB (H13^b) strains. With the B6 (H13^a) sequence as reference, identical residues are shown by dots and substitutions by the single letter code. The antigenic H13^a SVL9 peptide and its H13^b homolog SIL9 sequences are boxed.

data base. There was, however, significant similarity between the 47c1 sequence and several mouse and human EST clones (e.g., accession numbers W46128 and AA126873). These ESTs were derived from an array of diverse tissues representing placenta, embryo, adult brain, retina, thymus, and lymphoid cells. The 47c1 clone and these ESTs are also similar to hypothetical yeast and C. elegans 67.5 kd and 52.8 kd proteins, respectively (accession numbers P34248 and P49049). Thus, 47c1 encodes a novel, widely expressed protein whose only known function, as shown here, is to serve as a donor for the antigenic H13 peptide.

A Conservative Valine → Isoleucine Substitution Accounts For Self/Nonself Discrimination at the *H13*^{a} Locus

What was the explanation for the antigenic polymorphism of the *H13* locus? As seen by the Southern blot analysis above, both the *H13*^{a} and *H13*^{b} alleles were present in the appropriate mouse strains. Furthermore, both alleles were also found to be transcribed by Northern blot analysis (data not shown). To assess directly the antigenic reactivity of these allelic gene products, PCR primers were used to amplify genomic DNA flanking the region known to encode the H13^a antigen from mouse strains that represent both *H13*^{a} and *H13*^{b} haplotypes (see Experimental Procedures). The *H13*^{a} B6 and *H13*^{b} BALB (see below)-derived PCR fragments were subcloned into pcDNAI vector and tested for their ability to stimulate the H13^a-specific 30NX/B10Z T hybrid after transfection into D^b recipient cells. Significantly, although the expected PCR products were obtained from both the B6 and the BALB strains, only the PCR constructs from the B6 strain generated the T cell–stimulating ligand (Figure 6A). Thus, the antigenic polymorphism of the H13 alleles could be attributed to variation in their coding sequences.

To define precisely the amino acid sequence differences between the *H13 a* and *b* alleles, PCR fragments derived from the known H13^a strains (B6 and B10) and H13^b strains (B10.CE(30NX) and LP/J) as well as BALB/c were sequenced. Alignment of the predicted amino acid sequences is shown in Figure 6B. Most significantly,

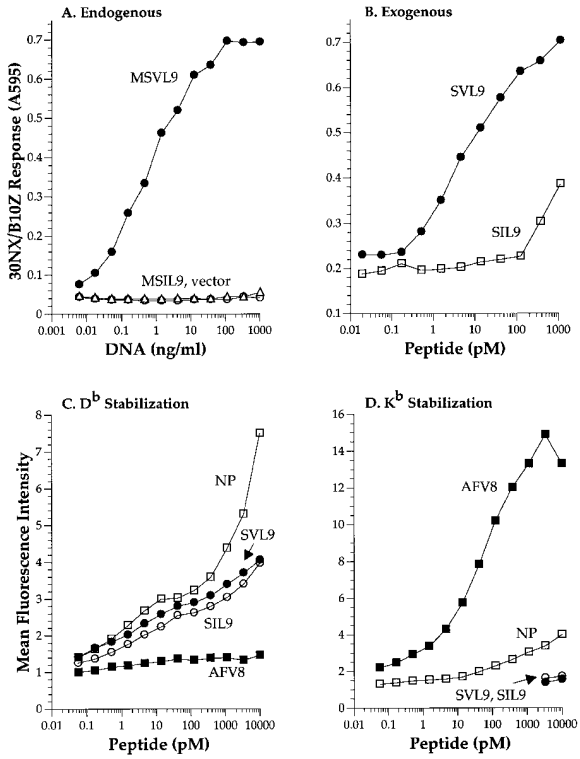

Figure 7. Endogenous and Exogenous Antigenic Activity and D^b MHC Binding Capacity of $H13^a$ and $H13^b$ Antigenic Homologs S[I,V]L9

(A) Endogenous generation of the 30NX/B10Z T cell–stimulating $H13/D^b$ ligand in APC transfected with varying amounts of DNA encoding the $H13^a$, MSVL9 or $H13^b$, MSIL9 sequences.

(B) T cell–stimulating activity of SVL9 versus SIL9 synthetic peptides added exogenously to D^b-L cells as APC.

(C, D) The D^b and K^b MHC stabilization capacity of SVL9, SIL9, the influenza NP (ASNENMETM) and the AFV8 (ANYDFICV) peptides (Malarkannan et al., 1996b). The TAP-RMA/S cells were incubated with varying concentrations of the indicated peptides and stained with either the anti-D^b or anti-K^b MAb. The NP and AFV8 peptides respectively serve as reciprocal controls to demonstrate the D^b and K^b MHC specificity of binding.

among the two to four amino acid substitutions found in this region, a single Val→Ile substitution was found within the SVL9 region (boxed in Figure 6B) and was consistent with expression of H13 antigen typing among these strains. Presence of this amino acid substitution within the *H13*^{a} residues suggested that this substitution could cause the observed failure of *H13*^{b} PCR constructs to generate the T cell ligand. To test directly the significance of the Val→Ile substitution, the Met SSVIGVWYL (MSIL9) expression construct was prepared and tested for activity. However, unlike the highly active MSVL9 construct, the MSIL9 construct failed to generate the T cell–stimulating ligand following transfection into D^b+ recipient cells (Figure 7A). We conclude that a single conservative Val→Ile substitution alone could give rise to the $H13^a$ antigen.

The inability of the MSIL9 expressing cells to stimulate the 30NX/B10Z T cell could have resulted from a failure to generate the SIL9/D^b complex via the intracellular antigen processing pathway, from an inability of the SIL9 peptide to bind D^b, or from a failure of the T cell to recognize the SIL9/D^b complex. To distinguish among these possibilities, the SIL9 peptide was synthesized and added exogenously to D^b APC, thus obviating the requirement for processing the endogenous precursor. Again, in contrast to the high potency of the SVL9 peptide, the SIL9 peptide was several hundred– to a thousand-fold less active (Figure 7B). To assess whether the difference in peptide specificity of the T cell response was due to decreased binding of SIL9 peptide to the D^b MHC, the peptides were tested for their ability to stabilize D^b MHC expression on the surface of TAP^- RMA/S cells. Significantly, both the SVL9 and the SIL9 peptides were comparable in their ability to stabilize D^b MHC but were consistently 3- to 10-fold less efficient than the prototype D^b MHC-binding influenza nucleoprotein (NP) peptide (Figure 7C). The peptide binding to the D^b MHC was specific because the AFV8 peptide that binds K^b (Malarkannan et al., 1996b) did not stabilize the D^b MHC. Conversely, only the AFV8 peptide stabilized the K^b MHC, and all three D^b binding peptides failed to stabilize K^b in the same experiment (Figure 7D). Thus, the explanation for the $H13^a$ antigenic polymorphism was not a failure to generate the peptide/D^b complexes, but instead the ability of the 30NX/B10Z T cell antigen receptor to discriminate between the valine versus the isoleucine side chains, a single methyl group substitution, within the SVL9 and the SIL9/D^b complexes. We subsequently refer to these ligands collectively as the S[I,V]L9/D^b complexes.

The S[I,V]L9 Peptides Are Immunodominant Epitopes in Reciprocal Anti-$H13^{a/b}$ CTL Responses

Graft rejection and D^b-restricted cytotoxic T lymphocytes (CTLs) can be elicited bidirectionally, in the $H13^a$ anti-$H13^b$ and the $H13^b$ anti-$H13^a$ directions (D. R., unpublished data). Considering that both S[I,V]L9 peptides could bind D^b, each peptide could serve as a potential T cell receptor (TCR) ligand and thus provide a molecular explanation for this reciprocal CTL activity. To test this hypothesis, CTL responses were elicited in the B10 (*H13*^{a}) and the B10.CE(30NX)-*H13*^{b} strains by reciprocal immunization. Bulk CTLs generated in mixed leukocyte culture were tested for their lytic specificity using the SVL9, the SIL9, or the NP peptide as a negative control. The SVL9 peptide sensitized T2-D^b target cells at a subpicomolar concentration for high levels of lysis by bulk anti-*H13*^{a} CTL cultures, demonstrating that the CTL activity was predominantly directed at the SVL9 epitope (Figure 8A). Again the SIL9 peptide was recognized by the anti-$H13^a$ CTL, but at a ~300-fold higher concentration. In repeated experiments, a half-maximal concentration of SIL9 versus SVL9 peptides for target cell lysis by the 30NX anti-B10 CTL ranged between 130- and 500-fold. Virtually identical results were observed when normal splenic lymphoblasts, rather than T2-D^b, were used as peptide-sensitized target cells (data not shown). We conclude that the anti-$H13^a$ CTLs demonstrate a strong but not absolute preference for the SVL9 peptide.

The reciprocal ($H13^a$ anti-$H13^b$) immunization also elicited CTLs that efficiently lysed target cells at a subpicomolar concentration, but instead with a strong prefer-

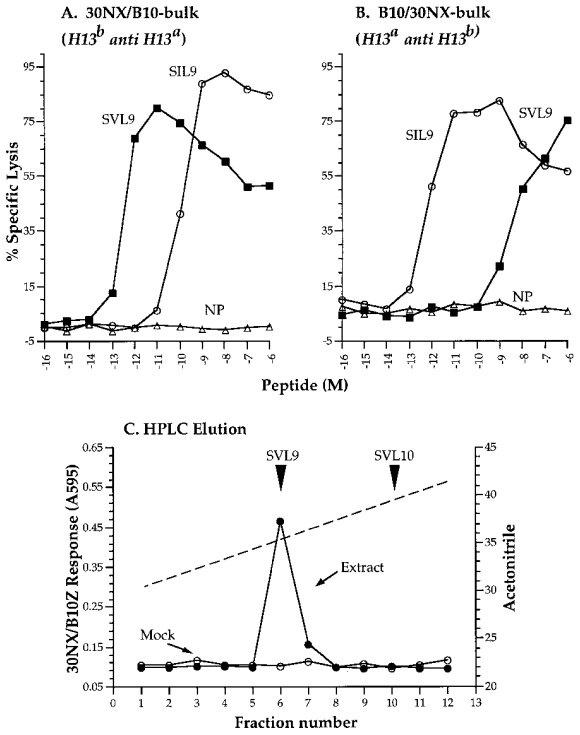

Figure 8. Bulk CTL Responses to H13^a and H13^b Preferentially Recognize the SVL9 and SIL9 Peptides, Respectively

(A) 30NX/B10-bulk CTLs, elicited by immunization of B10.CE(30NX)-*H13*^{b} with B10-*H13*^{a} cells and (B) B10/30NX-bulk CTLs, elicited by reciprocal immunization, were analyzed for their lytic specificity with T2-D^b targets in the presence of varying concentrations of SVL9 and SIL9 peptides. Specific lysis was determined at the indicated concentrations of the synthetic peptides at a 10:1 or 20:1 effector-to-target cell ratio.

(C) The naturally processed H13^a peptide coelutes with SVL9. A total EL-4 extract was fractionated by HPLC and the fractions assayed for the presence of 30NX/B10Z-stimulating activity using D^b-L cells as APC. Mock indicates fractions collected in an identical run with sample buffer alone prior to the extract sample. The arrows mark the retention time of SVL9 and SVL10 peptides under identical run conditions. The dashed line indicates the percent acetonitrile concentration.

ence for the SIL9 over the SVL9 peptide (Figure 8B). The SVL9 peptide that was active at subpicomolar concentrations for anti-H13^a CTL was now required at an approximately 5000-fold higher concentration to observe similar levels of target cell lysis by the anti-H13^b CTL. As expected, a CTL clone specific for the unrelated H3a^a antigen showed no lytic activity with either the SVL9 or the SIL9 peptides (data not shown), and none of the CTL responded to the NP peptide. We conclude that both the SVL9 and the SIL9 peptide/MHC complexes were presented efficiently by APC in vivo and were capable of eliciting CTL responses with reciprocal mirror image specificities. Intriguingly, with both anti-H13^a and anti-H13^b CTL, equivalent levels of lysis were obtained with the self-H13 homolog peptides, but at several hundred– to several thousand–fold higher concentrations.

Natural Processing of SVL9 in *H13*^{a} Cells

To determine the abundance and to establish the identity of the naturally processed H13 peptide as the SVL9 rather the SVL10 peptide that was also active (Figure 5C), we analyzed the acid soluble peptide pool in the EL-4 cell line that is derived from the *H13*^{a} B6 strain mice. The peptide mixture was fractionated by HPLC using a reverse phase C18 column, and each fraction was tested for 30NX/B10Z stimulating activity. This complex peptide pool contained only a single peak of T cell–stimulating activity that coeluted with the synthetic SVL9 peptide and was clearly distinguishable from the elution profile of the synthetic SVL10 peptide (Figure 8C). Comparison of the activity of the HPLC peak with a standard curve run in parallel, and based upon control spiking experiments showing that the experimental recovery of the SVL9 peptide ranged between 10–50% (data not shown), showed that EL-4 cells could express at most 45 copies/cell of this peptide. Judging by the somewhat higher T cell–stimulating activity of EL-4 relative to splenic blasts (Figure 1), as well as similar amounts of H13^a and H13^b transcripts in Northern blots (data not shown), we expect that the natural abundance of the S[I,V]L9 peptides is not likely to be any higher in normal cells. This result establishes the congruence between SVL9 and its naturally processed analog and clearly demonstrates that rare peptides can serve as minor histocompatibility antigens.

Discussion

Minor histocompatibility (*H*) loci are known to encode antigens responsible for inducing T cell responses, and thereby causing GVHD and/or chronic tissue rejection among individuals with identical MHC molecules. We show here that the T cell–stimulating activity of the mouse *H13* locus is due to the product of a novel chromosome 2 gene that can be defined solely by the conservative Val↔Ile substitution within the SSV[I/V]GVWYL (abbreviated as S[I,V]L9) nonapeptide/D^b MHC complexes. The results provide a molecular explanation for how antigenic polymorphism arises as a consequence of allelic variation at a classically defined autosomal minor *H* locus and further insights into the nature and immunological role of naturally processed minor H peptides.

The *H13* Minor Histocompatibility Locus

The products of the numerous autosomal minor *H* loci have long remained elusive, described at best by the approximate chromosomal locations of their genes or as T cell–stimulating antigenic activity in cell extracts (http://www.informatics.jax.org/encyclo.html; Wallny and Rammensee, 1990; Doolittle et al., 1996). Given the enormous complexity of the naturally processed peptide pool (Hunt et al., 1992), identifying the active peptide by biochemical purification is a daunting task that, even when accomplished, does not readily allow identification of the donor protein if there are no matches in the sequence data bases (den Haan et al., 1995; Perreault et al., 1996). As demonstrated here, expression cloning of the T cell–stimulating antigen genes represents an alternative and general strategy for identification of minor *H* loci, including those that yield rare peptide/MHC complexes. A clear advantage of the expression cloning strategy is that it allows direct identification of the gene

encoding the minor H peptide without the requirement for sophisticated instrumentation or prior knowledge of its chromosomal location. It is likely that this technique will facilitate the characterization of other minor H genes as well (N. S. and D. R., unpublished data).

The *H13* locus was originally identified by Snell and coworkers by the trait of tissue rejection and was functionally mapped to the central region of chromosome 2 between agouti, *a*, and another minor H locus, *H3* (Snell et al., 1967). Our mapping of the 47c1 cDNA in *H13* and *H3* congenic strains confirms Snell's chromosomal positioning at the molecular level and rules out an alternative trans-regulation model of genetic control at least for the *H13* minor antigen locus. Analysis of interspecific backcross panels described here positioned the *H13* locus such that it cosegregated with *a.* A mouse stock carrying a mutation affecting the *a* locus was reported to reject skin from wild-type mice, suggesting that this mutation could act as an *H* locus (McKenzie et al., 1985). While the relationship between this mutant *H* locus and *H13* remains unknown, the lack of any discernible sequence similarity between 47c1 cDNA and the *a* gene product makes it unlikely that *H13* is related to *a.* The *H13* locus thus represents a novel protein that, based upon similarity to EST cDNAs, appears to be expressed in diverse mammalian tissues and may also have homologs in other species. Although the only known function of the H13 protein is to serve as a donor for the SVL9/D^b complex, the availability of the cDNA clone should allow elucidation of its other cellular functions as well.

The H13/D^b Peptide Complex

The H13 nonapeptides S[I,V]L9 differ from all other peptides known to be presented by the D^b MHC molecule (Brusic et al., 1996). Previously defined antigenic peptides, such as those from the influenza nucleoprotein, ASNE**N**MET**M** (Falk et al., 1991a), the SV40 large T antigen, CKGV**N**EEY**L** (Lill et al., 1992), or the HY peptide, WMHH**N**MDL**I** (Greenfield et al., 1996), all share the nonamer consensus motif XXXX[**N**]XXX[**I,L,M**] defined by sequencing a mixture of D^b-bound peptides (Falk et al., 1991b). However, as shown here by the existence of S[I,V]L9/D^b complexes, the consensus motif clearly fails to predict all peptides that can specifically bind D^b MHC and stimulate potent T cell responses. Given that the consensus motif was identified by sequencing a mixture containing thousands of different peptides, it represents only the most abundant peptides that survive the isolation procedure and are amenable to microsequencing procedures. Peptides such as S[I,V]L9, which are rare and appear to bind D^b MHC less well, may not be recovered in sufficient amounts to yield strong signals characteristic of the asparagine anchor residue in the D^b motif. Note that the anchor residue(s) of the S[I,V]L9 peptides are not yet known, and it remains to be determined whether they define an alternate set of peptides with a novel consensus motif. Curiously, the $H13^a$ antigen is also expressed well in TAP^- RMA/S cells (D. C. R., unpublished observations), suggesting the interesting possibility that the H13 precursor could be processed in a manner distinct from conventional cytoplasmic precursors and may yield atypical peptides for binding to D^b MHC in the endoplasmic reticulum (ER). In either event, analysis of how the S[I,V]L9 peptides are generated and presented by D^b MHC should provide additional insights into the antigen presentation pathway.

Allelic Polymorphism of *H13* Locus and Its Role in the Immune Response

The similarity between the self and foreign *H13* peptides is striking. Both allelic S[I,V]L9 homologs bound D^b MHC equivalently, stimulated their respective CTLs at similar concentrations, and elicited potent CTL responses. The presence or absence of the single methyl group (valine versus isoleucine) in the S[I,V]L9/D^b complex thus provides the primary structural basis for the self/nonself discrimination by the T cell repertoire. Interestingly, a single conservative amino acid substitution (Ile 6 to Ala) was also found to distinguish the mitochondrial H antigen alleles, the ND1α and the ND1β peptides presented by the H2-M3 class 1b MHC molecule (Loveland et al., 1990), and the allelic ATPase6 peptides (Glu to Lys) presented by the rat MHC class I (Bhuyan et al., 1997). While it would be tempting to conclude that these substitutions affect T cell specificity because they contact the TCR, note that the recently solved crystal structure of the ND1 peptide/H2-M3 structure showed that the variant p6 Ile residue was solvent *in*accessible (Wang et al., 1995a). Thus, amino acid substitutions in the antigenic peptide could also affect specificity of the T cell response without directly contacting the TCR. Because the S[I,V]L9 peptides lack the D^b consensus motif, the rules of solvent accessibility of individual side chains based upon the recently solved crystal structures of the NP peptide/D^b complex (Young et al., 1994) cannot be extrapolated to predict the orientation and presumed TCR accessibility of the p4 Val versus the Ile residues. This problem can, however, be resolved by determining the crystal structures of the S[I,V]L9/D^b complexes.

A number of hypotheses have been proposed to explain the immunogenicity of minor *H* gene products (reviewed in Lindahl, 1991; Roopenian, 1992; Goulmy, 1996). The simplest of these falls under the rubric of determinant selection, in which amino acid substitutions in the *H* gene product in the donor strain cause presentation of novel minor H peptide/MHC complexes that are absent in the recipient strain and therefore immunogenic. This model does explain the mouse HY-specific T cell response: female mice not only lack the Y chromosome–derived *Uty*- and *Smcy*-derived peptide/MHC complexes but also fail to present homologous peptides (encoded by the X chromosome homologs *Utx* and *Smcx*), due to a deletion or a lack of MHC binding (Scott et al., 1995; Greenfield et al., 1996). The same model may also be applicable to the *Smcy*-encoded T cell epitopes in humans (Wang et al., 1995b; Meadows et al., 1997). The male cell surface is thus unambiguously foreign to the female T cells.

Unlike the mechanism of antigenic polymorphism at the *HY* loci, T cell recognition of the allelic H13 peptides lies on the cusp of foreign- versus self-reactivity. Although both anti-$H13^a$ and anti-$H13^b$ CTL clearly preferred the foreign peptide, they also cross-reacted with the self-H13 homologs (Figure 8). In particular, for the

anti-H13^a CTL, equivalent levels of lysis were observed with the foreign SVL9 and the self-SIL9 peptide at a few hundred–fold higher concentration. The S[I,V]L9 peptides should therefore be considered partial and reciprocal agonists that define the limits of negative selection. Given that T cell activation requires the presence of only a few peptide/MHC complexes (Kageyama et al., 1995; Malarkannan et al., 1996a), the escape from negative selection of the anti-H13 TCRs would be possible only if the natural abundance of the self-S[I,V]L9/D^b complexes remained below ~100–1000 copies/cell. The measurement of naturally processed SVL9 peptide at ~50 copies/cell is in accordance with this expectation. Whether exceeding this "safe limit" will cause H13-specific tolerance or autoimmunity remains an interesting question.

The existence of partial H13 agonists also suggests another role for autosomal peptides during development of the T cell repertoire that could explain their potent immunogenicity. The expression of H13 transcripts in the spleen as well as in the thymus (data not shown) provides an opportunity for the S[I,V]L9/D^b complexes to participate in positive selection of the TCR repertoire as well. Thus, as demonstrated in model systems, peptide-specific, low affinity interactions with the self-H13 peptide/D^b complex could favor positive selection of TCRs specific for the alternative allelic homolog (Ashton-Rickardt et al., 1993; Hogquist et al., 1994, 1997; Sebzda et al., 1994; Pawlowski et al., 1996), which in turn could impact upon the frequency and/or TCR affinity of the precursor CTLs that emerge. Such a scenario might explain why T cell responses to single autosomal minor H antigens are high in magnitude and why responses to "merely foreign" HY antigens can be readily competed out by the autosomal "neo-self" H antigens (Wettstein, 1986). This novel hypothesis as well as the role of minor *H* antigens in tissue rejection reactions can now be examined directly.

Experimental Procedures

Mice

All the indicated inbred mouse strains were obtained from or bred at The Jackson Laboratory (Bar Harbor, ME). (BALB/c × BALB.B)F1 mice were bred in the animal care facility at the University of California (Berkeley, CA) from Jackson Laboratory stocks.

Cell Lines

Cell lines were maintained in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS; Hyclone, Logan, UT) or in Dulbecco's modified Eagle's medium (DMEM) supplemented as described (Cerottini et al., 1974). LMtk-(C3H, H-2^k) and its MHC-expressing transfectant derivatives have been described previously (Shastri et al., 1985). The D^{b}L-B7 transfectant was generated by transfection of D^{b}L with a B7–2 cDNA construct (a gift from J. Allison, University of California, Berkeley, CA) and selection with 1 mg/ml G418. EL4-B7 (H-2^b), RMA (H-2^b), and its TAP-derivative RMA/S cell lines were obtained from Drs. J. Allison and D. Raulet (University of California, Berkeley, CA). T2-D^b cells were a kind gift of P. Cresswell (Yale University, New Haven, CT). Splenic blasts were made by stimulating spleen cells for 2 days with 4 μg/ml concanavalin A and 30 μg/ml lipopolysaccharide. The H13^a-specific 30NX/B10–1 clone CTL line was generated by immunizing the B10.CE (30NX)-*H13^b*/Sn mice with cells from the B10-H13^a (H13^a) mice as described (Cariappa et al., 1996). The H3a^a-specific, D^b-restricted cloned CTL line Lpa/2R-1 (formerly C1) and the K^b-restricted β2M^b-specific cloned CTL line F1/5R-5 (formerly C2) have been described (Roopenian and Davis, 1989). All CTL lines were maintained by weekly stimulation with B10 or B6 spleen cells and 10–30 U/ml recombinant interleukin-2 (rIL-2) using established conditions (Roopenian and Davis, 1989). The corresponding *lac*Z inducible T cell hybrids, 30NX/B10Z, Lpa/2RZ, and F1/5R-5Z, were generated by fusing the CTL lines with the BWZ.36/CD8α fusion partner as described (Sanderson and Shastri, 1994). The anti-H-2^b alloreactive 18.5Z T cell hybrid has been previously described (Malarkannan et al., 1995a). To generate H13^b anti-H13^a CTL, B10.CE(30NX)-*H13^b*/Sn mice were primed twice with 2 × 10^7 spleen cells from male B10 mice and then restimulated for 5–6 days with spleen cells from 200 Gy-irradiated mice in DMEM medium supplemented on day 3 of culture with 10–30 U/ml rIL-2 as described (Roopenian et al., 1993). 30NX/B10-bulk is a cryopreserved pool of four such MLCs used after a 6-day expansion with B10 spleen cells and 30 U/ml rIL-2. To generate H13^a anti-H13^b CTL, female B10 mice were primed with male B10.CE(30NX)-H13^b/Sn spleen cells and then grafted with tail skin from female B10.CE(30NX)-*H13^b*/Sn mice. When rejection of grafts was occurring, peripheral blood leukocytes were obtained from retro orbital blood, purified, and cultured in MLC with irradiated female 30NX cells and 30 U rIL-2 for 9 days as described (Roopenian et al., 1993). B10/30NX-bulk is such a culture restimulated once after MLC with irradiated 129/J (H13^b) cells and 30 U/ml rIL-2.

T Cell Activation Assays

T cell responses specific for peptide/MHC were measured by the production of β-galactosidase activity in the T cell hybrids (Karttunen and Shastri, 1991; Sanderson and Shastri, 1994). T hybrids (3–10 × 10^4) were cocultured overnight with APC (2–5 × 10^4) either expressing the antigen endogenously, transfected with antigen cDNAs, or with exogenous peptides in 96-well plates. The peptide/MHC-induced T cell response was determined using the β-galactosidase substrate chlorophenol red β-galactoside (CPRG) as described (Sanderson and Shastri, 1994). The conversion of CPRG to chlorophenol red was measured at 595 nm and 655 nm as a reference wavelength with a 96-well microplate reader (BioRad, Richmond, CA). Data show the mean absorbence of replicate cultures and are representative of at least three independent experiments. Chromium release assays for CTL activity were a modification of that described earlier (Roopenian et al., 1983). Peptide pulsed target cells T2-D^b were prepared by adding 2–5 × 10^4 ^{51}Cr-labeled target cells to V-bottom microtiter wells with varying peptide concentrations for 1 hr. Effector cells were then added at an effector-to-target ratio of 10:1 or 20:1 and incubated for 4 hr. Percent specific lysis was calculated from the amount of ^{51}Cr released into the culture supernatant and is shown as the mean of triplicate cultures.

cDNA Library and Expression Screens

A unidirectional cDNA library was constructed (Superscript Choice System, GIBCO-BRL) using poly (A)+ mRNA from EL4 cells in the BstXI–NotI sites of the mammalian expression vector pcDNA1 (Invitrogen, San Diego, CA; Malarkannan et al., 1995a, 1996a). The cDNAs were screened by transforming competent bacteria with recombinant plasmids and culturing in pools of ~30–100 cfu in 96-well U-bottom plates. For expression screens, aliquots of cDNA (50–100 cfu/well) prepared directly in the 96-well plates were transiently transfected into 3 × 10^4 LMtk cells cotransfected with the relevant MHC class I cDNA (10 ng/ml) and B7-2 cDNA (5 ng/ml). Two days later, 10 × 10^4 30NX/B10Z T cells were added per well and cocultured overnight. Positive pools were identified by adding CPRG and scoring pools with above background absorbence. The single plasmid encoding the antigenic activity was identified by repeating the screen with individual colonies obtained from the positive cDNA pool. The sequence of 47c1 cDNA is available from the NCBI GenBank with the accession number AF017785.

Expression Constructs and Peptides

Deletion constructs were generated by amplifying 47c1 DNA with a vector-specific forward primer and a 47c1 reverse primer R4 (5′-CTGCCCTTGTCAGCCCAACCTC-3′), R7 (5′-TCAGAAGGTACCAG ACACCA-3′), R8 (5′-AGTGTCAAACTCATAGTTGA-3′), or R11 (5′-TAGGCCCAGGCACACCAGGT-3′) using Pfu polymerase (Stratagene,

La Jolla, CA). PCR fragments were digested with BamHI in the 5′ flanking region of the vector and cloned into the BamHI–EcoRV sites of pcDNA1. The F1ΔR1 construct was made similarly except that F1 (5′-TACCGAGCTCGGATCCCTCTGCTTATGAAACGTTAC-3′) containing an internal BamHI site was used as the forward primer and R1 (5′-AAGACCAGCTGAGGGCATCTG-3′) was the reverse primer. Minigene constructs MSVL10 (MSSVVGVWYLL), MSVL9 (MSSVVGVWYL), MSIL9 (MSSVIGVWYL) Db1 (LNL9), and Db2 (PND9) shown in Figures 4 and 5 were prepared using complementary oligonucleotides corresponding to the indicated sequences with an ATG codon at the 5′ end for translation initiation and a termination codon at the 3′ end. Deletion constructs from BALB/c and B6 genomic DNA were generated by PCR using the F1 and R1 or R4 primers as described above, and the amplified fragments were cloned into the BamHI–EcoRV sites of pcDNA1 as above. The synthetic peptides, SSVVGVWYLL (SVL10), SSVVGVWYL (SVL9), SSVIGVWYLL (SIL10), and SSVIGVWYL (SIL9) were prepared using solid-phase F-Moc chemistry on the ABI Model 421 synthesizer, purified by HPLC, and confirmed by mass spectrometry. The peptides ANYDFICV (AFV8) and ASNENMETM (NP) presented by K^b and D^b MHC to appropriate T cells have been described (Shastri and Gonzalez, 1993; Malarkannan et al., 1996b).

Genetic Mapping

For Southern blot RFLP analysis, splenic genomic DNA purchased from the Jackson Lab DNA resource, or liver DNA from C57/BL10SnJ (B10), CE/J, B10.CE (30NX)-*H13*^{b}/Sn, LP/J, B10.LP-*H13*^{b}*A*^{w}/Sn, B10.LP/Sn, and B10.LP-*H3*^{b} mice was digested with XbaI prior to electrophoresis and transfer to nylon membranes (Zeta-bind, Bio-Rad). The blot was probed with ^{32}P-labeled 1.3 kb DNA fragment of 47c1 containing the antigenic peptide. The 47c1 clone was mapped on the Jackson Laboratory BSS and BSB DNA panels after identification of a SSLP between the parental strains B6 and SPRET/Ei. The primer pairs H13-F (5′-AGCTCCCTTGGAGCACGGTGG-3′) and H13-R (5′-GCTCCTCCTATCCCTGCAGGC-3′) amplify a 163 bp genomic DNA fragment from the 3′ untranslated region of the 47c1 gene. MDE gels were run according to the manufacturers' suggested protocols (FMC BioProducts, Vallensbaek, Denmark), and the SSLP was visualized by autoradiography.

RMA/S Stabilization and FACS Analysis

RMA/S cells were cultured overnight at 31°C and then for 60 min with varying concentrations of the indicated synthetic peptides. The cells were washed twice with phosphate-buffered saline (PBS) then cultured for 4 hr at 37°C. The cells from each sample were split in half and stained with either the anti-K^b (5F1) or the anti-D^b (B22.241R) MAb followed with fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody. The cells were washed twice in PBS/FBS and analyzed by fluorescence-activated cell sorter.

Extraction and HPLC Analysis of Naturally Processed Peptides

Total acid soluble peptide pool from EL4 cells was extracted as described (Rotzschke et al., 1990b; Malarkannan et al., 1995b). Briefly, 8×10^7 EL-4 cells were washed with PBS and extracted with 1 ml of 10% boiling acetic acid for 5 min. Cellular debris was removed by centrifugation and fractionated by HPLC after filtration through a 10 kd filter. Reverse-phase C18 column (Vydac, 4.6 × 250 mm, 5 μm, 300 Å) was run in 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Flow rate was maintained at 1 ml/min, and 1 ml fractions were collected, dried in a vacuum centrifuge, and resuspended in 50 μl of PBS+10% dimethyl sulfoxide. Serial dilutions of each fraction were assayed for stimulating 30NX/B10Z T cells in a total volume of 200 μl as described above. Mock injections with sample buffer alone, were performed prior to each extract sample using the same column and identical run conditions, to demonstrate absence of cross-contamination between samples. The collected fractions were assayed in the same experiment, using the same APC and T cells, in parallel with fractions from the cell extracts and synthetic peptide standards. The minimal SVL9 peptide concentration required for 30NX/B10Z activation was typically in the range of 100–1000 fM. The abundance of naturally processed peptides was determined by comparison with the synthetic peptide standard curve and recovery estimates from spiking cells with a known amount of peptide and back-extraction.

Acknowledgments

We are grateful to Mr. Federico Gonzalez and Mr. Arta Monir Monjazeb for technical assistance and to Dr. Astar Winoto and Ms. Jessica Chubak for comments on the manuscript. This research was supported by grants from the National Institutes of Health (NIH) to N. S. and D. R.; L. M. was supported by the University of California Presidents Postdoctoral Fellowship and an NIH Training grant.

Received June 26, 1997; revised August 20, 1997.

References

Allen, P.M. (1994). Peptides in positive and negative selection: a delicate balance. Cell *76*, 593–596.

Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N.M., Ploegh, H.L., and Tonegawa, S. (1993). Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. Cell *73*, 1041–1049.

Auchincloss, H., and Sultan, H. (1996). Antigen processing and presentation in transplantation. Curr. Opin. Immunol. *8*, 681–687.

Bevan, M.J. (1975). Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. Nature *256*, 419–421.

Bhuyan, P.K., Young, L.L., Fischer Lindhal, K., and Butcher, G.W. (1997). Identification of the rat maternally transmitted minor histocompatibility antigen. J. Immunol. *158*, 3753–3760.

Brusic, V., Rudy, G., Kyne, A.P., and Harrison, L.C. (1996). MHCPEP—a database of MHC-binding peptides: Update 1995. Nucleic Acids Res. *24*, 242–244.

Cariappa, A., Flyer, D.C., Rollins, C.T., Roopenian, D.C., Flavell, R.A., Brown, D., and Waneck, G.L. (1996). Glycosylphosphatidylinositol-anchored H-2D-b molecules are defective in antigen processing and presentation to cytotoxic T lymphocytes. Eur. J. Immunol. *26*, 2215–2224.

Cerottini, J.C., Engers, H.D., Macdonald, H.R., and Brunner, T. (1974). Generation of cytotoxic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. J. Exp. Med. *140*, 703–717.

Dabhi, V.M., and Lindahl, K.F. (1996). CTL respond to a mitochondrial antigen presented by H2-D-b. Immunogenetics *45*, 65–68.

den Haan, J.M.M., Sherman, N.E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J.W., Skipper, J., Shabanowitz, J., Hunt, D.F., Engelhard, V.H., and Goulmy, E. (1995). Identification of a graft versus host disease-associated human minor histocompatibility antigen. Science *16718*, 20479–21077.

Doolittle, D.P., Davisson, M.T., Guidi, J.N., and Green, M.C. (1996). Catalog of mutant genes and polymorphic loci. In Genetic Variants and Strains of the Laboratory Mouse, M.F. Lyon, S. Rastan, and S.D.M. Brown, eds. (Oxford, U.K.: Oxford University Press), pp. 17–854.

Falk, K., Rotzschke, O., Deres, K., Metzger, J., Jung, G., and Rammensee, H.-G. (1991a). Identification of naturally processed viral nonapeptides allows their quantitation in infected cells and suggests an allele specific T cell epitope forecast. J. Exp. Med. *174*, 425–434.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991b). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*, 290–296.

Goulmy, E. (1996). Human minor histocompatibility antigens. Curr. Opin. Immunol. *8*, 75–81.

Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J.H.F., Vossen, J., Gratwohl, A., Vogelsang, G.B., Van Houwelingen, H.C., and Van Rood, J.J. (1996). Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N. Engl. J. Med. *334*, 281–285.

Graff, R.J., Brown, D.H., and Snell, G.D. (1977). The alleles of the *H-13* locus. Immunogenetics *7*, 413–423.

Greenfield, A., Scott, D., Pennisi, D., Ehrmann, I., Ellis, P., Cooper, L., Simpson, E., and Koopman, P. (1996). An H-YD^b epitope is encoded by a novel mouse Y chromosome gene. Nature Genet. *14*, 474–478.

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17–27.

Hogquist, K.A., Tomlinson, A.J., Kieper, W.C., McGargill, M.A., Hart, M.C., Naylor, S., and Jameson, S.C. (1997). Identification of a naturally occurring ligand for thymic positive selection. Immunity *6*, 389–399.

Hunt, D.F., Henderson, R.A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A.L., Appella, E., and Engelhard, V.H. (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science *255*, 1261–1263.

Jameson, S.C., and Bevan, M.J. (1995). T cell receptor antagonists and partial agonists. Immunity *2*, 1–11.

Jameson, S.C., Hogquist, K.A., and Bevan, M.J. (1995). Positive selection of thymocytes. Annu. Rev. Immunol. *13*, 93–126.

Jardetzky, T.S., Lane, W.S., Robinson, R.A., Madden, D.R., and Wiley, D.C. (1991). Identification of self peptides bound to purified HLA-B27. Nature *353*, 326–329.

Kageyama, S., Tsomides, T.J., Sykulev, Y., and Eisen, H.N. (1995). Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. J. Immunol. *154*, 567–576.

Karttunen, J., and Shastri, N. (1991). Measurement of ligand induced activation in single viable T-cells using the lacZ reporter gene. Proc. Natl. Acad. Sci. USA *88*, 3972–3976.

Karttunen, J., Sanderson, S., and Shastri, N. (1992). Detection of rare antigen presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc. Natl. Acad. Sci. USA *89*, 6020–6024.

King, T.R., Christianson, G.J., Mitchell, M.J., Bishop, C.E., Scott, D., Ehrmann, I., Simpson, E., Eicher, E.M., and Roopenian, D.C. (1994). Deletion mapping by immunoselection against the H-Y histocompatibility antigen further resolves the Sxra region of the mouse Y chromosome and reveals complexity of the Hya locus. Genomics *24*, 159–168.

Lill, N.L., Tevethia, M.J., Hendrickson, W.G., and Tevethia, S.S. (1992). Cytotoxic T lymphocytes (CTL) against a transforming gene product select for transformed cells with point mutations within sequences encoding CTL recognition epitopes. J. Exp. Med. *176*, 449–457.

Lindahl, K.F. (1991). Minor histocompatibility antigens. Trends Genet. *7*, 219–224.

Little, C.C. (1914). A possible mendelian explanation for a type of inheritance apparently non-Mendelian in nature. Science *40*, 904–906.

Little, C.C. (1941). The genetics of tumor transplantation. In Biology of the Laboratory Mouse, G.D. Snell, ed. (Philadelphia: Blakiston), pp. 279–309.

Loveland, B.E., and Lindahl, K.F. (1991). The definition and expression of minor histocompatibility antigens. In Antigen Processing and Recognition, J. McCluskey, ed. (Boca Raton, FL: CRC Press), pp. 173–192.

Loveland, B., Wang, C.-R., Yonekawa, H., Hermel, E., and Lindahl, K.F. (1990). Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. Cell *60*, 971–980.

Malarkannan, S., Afkarian, M., and Shastri, N. (1995a). A rare cryptic translation product is presented by K^b MHC class I molecule to alloreactive T-cells. J. Exp. Med. *182*, 1739–1750.

Malarkannan, S., Goth, S., Buchholz, D.R., and Shastri, N. (1995b). The role of MHC class I molecules in the generation of endogenous peptide/MHC complexes. J. Immunol. *154*, 585–598.

Malarkannan, S., Gonzalez, F., Nguyen, V., Adair, G., and Shastri, N. (1996a). Alloreactive CD8+ T-cells can recognize unusual, rare and unique processed peptide/MHC complexes. J. Immunol. *157*, 4464–4473.

Malarkannan, S., Serwold, T., Nguyen, V., Sherman, L.A., and Shastri, N. (1996b). The mouse mammary tumor virus env gene is the source of a CD8+ T-cell-stimulating peptide presented by a major histocompatibility complex class I molecule in a murine thymoma. Proc. Natl. Acad. Sci. USA *93*, 13991–13996.

McKenzie, I.F., Henning, M.M., and Curnow, K.M. (1985). A new H gene associated with a coat color mutation. Transplant. Proc. *18*, 754–755.

Meadows, L., Wang, W., den Haan, J.M.M., Blokland, E., Reinhardus, C., Drijfhout, J.W., Shabanowitz, J., Pierce, R., Agulnik, A.I., Bishop, C.E., Hunt, D.F., Goulmy, E., and Engelhard, V.H. (1997). The HLA-A*0201-Restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. Immunity *6*, 273–281.

Morse, M.-C., Bleau, G., Dabhi, V.M., Hetu, F., Drobetsky, E.A., Lindahl, K.F., and Perreault, C. (1996). The COI mitochondrial gene encodes a minor histocompatibility antigen presented by H2-M3. J. Immunol. *156*, 3301–3307.

Pawlowski, T.J., Singleton, M.D., Loh, D.Y., Berg, R., and Staerz, U.D. (1996). Permissive recognition during positive selection. Eur. J. Immunol. *26*, 851–857.

Perreault, C., Jutras, J., Roy, D.C., Filep, J.G., and Brochu, S. (1996). Identification of an immunodominant mouse minor histocompatibility antigen (MiHA): T cell response to a single dominant MiHA causes graft-versus-host disease. J. Clin. Invest. *98*, 622–628.

Roopenian, D.C. (1992). What are minor histocompatibility loci? A new look at an old question. Immunol. Today. *13*, 7–10.

Roopenian, D.C., and Davis, A.P. (1989). Responses against antigens encoded by the H-3 histocompatibility locus: antigens stimulating class I MHC- and class II MHC-restricted T cells are encoded by separate genes. Immunogenetics *30*, 335–343.

Roopenian, D.C., Widmer, M.B., Orosz, C.G., and Bach, F.H. (1983). Response against single minor histocompatibility antigens. I. Functional and immunogenetic analysis of cloned cytolytic T cells. J. Immunol. *131*, 2135–2140.

Roopenian, D.C., Davis, A.P., Christianson, G.J., and Mobraaten, L.E. (1993). The functional basis of minor histocompatibility loci. J. Immunol. *151*, 4595–4605.

Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H.-G. (1990a). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature *348*, 252–254.

Rotzschke, O., Falk, K., Wallny, H.-J., Faath, S., and Rammensee, H.-G. (1990b). Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. Science *249*, 283–287.

Sanderson, S., and Shastri, N. (1994). LacZ inducible peptide/MHC specific T-hybrids. Int. Immunol. *6*, 369–376.

Scott, D.M., Ehrmann, I.E., Ellis, P.S., Bishop, C.E., Agulnik, A.I., Simpson, E., and Mitchell, M.J. (1995). Identification of a mouse male-specific transplantation antigen, H-Y. Nature *376*, 695–698.

Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S.M., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. Science *263*, 1615–1618.

Shastri, N. (1996). Needles in haystacks: identifying specific peptide antigens for T-cells. Curr. Opin. Immunol. *8*, 271–277.

Shastri, N., and Gonzalez, F. (1993). Endogenous generation and presentation of the OVA peptide/K^b complex to T-cells. J. Immunol. *150*, 2724–2736.

Shastri, N., Malissen, B., and Hood, L. (1985). Ia-transfected L-cell fibroblasts present a lysozyme peptide but not the native protein to lysozyme-specific T cells. Proc. Natl. Acad. Sci. USA *82*, 5885–5889.

Snell, G.D. (1948). Methods for the study of histocompatibility genes. J. Genet. *49*, 87–108.

Snell, G.D., Cudkowicz, G., and Bunker, H.P. (1967). Histocompatibility genes of mice. VII. H-13, a new histocompatibility locus in the fifth linkage group. Transplantation *5*, 492–503.

Van Bleek, G.M., and Nathenson, S.G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. Nature *348*, 213–216.

von Boehmer, H., and Kisielow, P. (1990). Self-nonself discrimination by T cells. Science *248*, 1369–1373.

Wallny, H.-J., and Rammensee, H.-G. (1990). Identification of classical minor histocompatibility antigen as cell-derived peptide. Nature *343*, 275–278.

Wang, C.R., Castano, A.R., Peterson, P.A., Slaughter, C., Lindahl, K.F., and Deisenhofer, J. (1995a). Nonclassical binding of formylated peptide in crystal structure of the MHC class Ib molecule H2-M3. Cell *82*, 655–664.

Wang, W., Meadows, L.R., den Haan, J.M.M., Sherman, N.E., Chen, Y., Blokland, E., Shananowitz, J., Agulnik, A.I., Hendrickson, R.C., Bishop, C.E., Hunt, D.F., Goulmy, E., and Engelhard, V.H. (1995b). Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. Science *269*, 1588–1590.

Wang, R.-F., Parkhurst, M.R., Kawakami, Y., Robbins, P.F., and Rosenberg, S.A. (1996). Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. J. Exp. Med. *183*, 1131–1140.

Wettstein, P.J. (1986). Immunodominance in the T cell response to multiple non-H-2 histocompatibility antigens. III. Single histocompatibility antigens dominate the male antigen. J. Immunol. *137*, 2073–2079.

York, I.A., and Rock, K.L. (1996). Antigen processing and presentation by the class I major histocompatibility complex. Annu. Rev. Immunol. *14*, 369–396.

Young, A.C.M., Zhang, W., Sacchettini, J.C., and Nathenson, S.G. (1994). The three-dimensional structure of H-2D^b at 2.4 Å resolution: implications for antigen-determinant selection. Cell *76*, 39–50.